



Mitochondrial data unravels the human-mediated introduction of *Mediodactylus kotschy* (Squamata: Gekkonidae) to the Central Balkan Peninsula

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Abstract. Kotschy’s gecko, *Mediodactylus kotschy*, is native to the Eastern part of the Mediterranean Basin and a successful colonizer, with an extensive distribution across the Balkans and outside its native range. In Serbia, it was presumed native in Prizren (Kosovo and Metohija province) and introduced across the remaining territory. To explore the origin of Serbian Kotschy’s gecko, we collected specimens from most of the Serbian populations where the species was recorded and assessed their phylogenetic relationships based on three mtDNA markers (COI, cytb, 16S rRNA) under Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. First, our results support that the specimens found in Serbia belong to *M. kotschy*. Second, all populations except Prizren belong to the subspecies *M. kotschyi skopjensis*. Furthermore, the Italian populations are sister to the specimens from Prizren, suggesting a Balkan origin for these populations, which settled in Apulia likely through ancient human-mediated dispersion. The small genetic differentiation found suggests that Kotschy’s gecko rapidly colonized the Central Balkans, originating from Northern Greece and North Macedonia. The results also suggest a story of multiple, complex (most likely) human-mediated introductions. However, further studies should be conducted to properly elucidate the colonization pathways, and to truly disentangle the potential fast natural range expansion from an anthropogenic dispersion of this gecko species.

Keywords: Balkans, Gekkota, introduced species, *Mediodactylus kotschy*, phylogeny.

Introduction

The Mediterranean Basin stands out for its extensive timeline of human settlements and commerce, as well as the ongoing interaction between humans and biodiversity, resulting

in numerous biological introductions spanning millennia (e.g., *Hemidactylus turcicus* in Rato et al. (2011), *Tarentola mauritanica* in Rato et al. (2010, 2021, 2023), and Deso et al. (2020)). Specifically, the Balkan Peninsula has histori-

cally served as a crucial crossroad, connecting the paths between Europe and Asia. Not surprisingly, there are a few records of anthropogenic reptile introductions, such as *Podarcis siculus* in Albania, Montenegro, Greece, Turkey, France, the Iberian Peninsula, North Africa and some Mediterranean islands (Silva-Rocha et al., 2014; Mizsei et al., 2016; Gvozdenović and Iković, 2022). Likewise, *Trachemys scripta* was introduced in Serbia, Montenegro, Croatia, Bulgaria, Greece, and many other countries of the Balkan Peninsula (Urošević, 2014; Jelić and Jelić, 2015; Tzankov et al., 2015; Stănescu et al., 2017; Koren et al., 2018; Tzoras et al., 2018; Urošević et al., 2019; Kornilev et al., 2020; Panjković et al., 2021; Ljubisavljević, 2022), but also amphibians such as *Xenopus laevis* and *Lithobates catesbeianus* (Panjković et al., 2021).

Fortunately, advances in molecular genetics and phylogeographic tools have significantly enhanced our understanding of invasion dynamics and the population characteristics of biological invasions, enabling us to determine the origin and patterns of introduction more accurately (Le Roux and Wicczorek, 2009). However, in situations where the genetic diversity is limited and the divergence is shallow, the distribution of the species can be attributed to either human introductions or recent natural colonization by very small propagules (e.g., Salvi et al., 2011; Silva-Rocha et al., 2012; Graciá et al., 2013; Bisbal-Chinesta et al., 2020).

Kotschy's gecko, *Mediodactylus kotschy* (Squamata: Gekkonidae), is a small crepuscular or nocturnal gecko considered as endemic to Europe, present in Greece, North Macedonia and possibly parts of Bulgaria, as a native species (see in Kotsakiozi et al., 2018; fig. 1a). It is found in dry rocky habitats under a Mediterranean climate, often associated with human buildings (Böhme et al., 2009; Urošević et al., 2016). This species exhibits a high morphologic diversity, which was initially used to describe at least 25 subspecies (Szczerbak, 1960; Baran and Gruber, 1982; Szczerbak and

Golubev, 1986; but see Uetz, 2024). However, the taxonomy of *M. kotschy* has been reshaped several times during the last decades. Recent research showed and confirmed the monophyly of the *Mediodactylus* genus (Macey et al., 2000; Bauer et al., 2013). Also, preliminary molecular studies questioned and disproved the taxonomy and the subspecies delimitations of *M. kotschy* (Kasapidis et al., 2005). Kotsakiozi et al. (2018) suggested through molecular species delimitation analyses that *M. kotschy* was in fact a species-complex composed of five taxa, and proposed the following taxonomy: *M. kotschy* (mainland Balkans, part of the Pannonian Plain, most of Aegean islands, and Italy; clade A), *M. orientalis* (Levant, Cyprus, southern Anatolia, and south-eastern Aegean islands; clade B), *M. danilewskii* (Black Sea region and south-western Anatolia; clade C), *M. bartoni* (Crete; clade D), and *M. oertzeni* (southern Dodecanese Islands; clade E). This taxonomy has been recently validated by Kotsakiozi et al. (2024) through genomic analyses. The *M. kotschy* species complex is thought to have diversified during the middle Miocene due to vicariance events, with the first differentiation dated to ~10-15 Mya. Most of the major clades diversified during the last 5 million years (Kasapidis et al., 2005; Kotsakiozi et al., 2018).

Mediodactylus kotschy is considered introduced to southwest Italy (Apulia and Basilicata), Serbia, Albania (coastal region from Shkoder to Vlore) and Hungary (Kotsakiozi et al., 2018; Babocsay, 2021). Furthermore, the species seems to be able to rapidly form colonies when introduced: a release in 1985 in Hungary caused the formation of a stable population recorded in 1998 (Farkas et al., 1999). In Serbia, only one population is currently assumed to be native or historical, located in Prizen (Ajtić and Tomović, 2001). Otherwise, it is considered as introduced and successfully established in Novi Sad (Ajtić, 2009; Urošević et al., 2021), Belgrade (Ajtić, 2009; Urošević et al., 2016, 2021), Smederevo (Urošević et al., 2016), and more recently confirmed in Niš

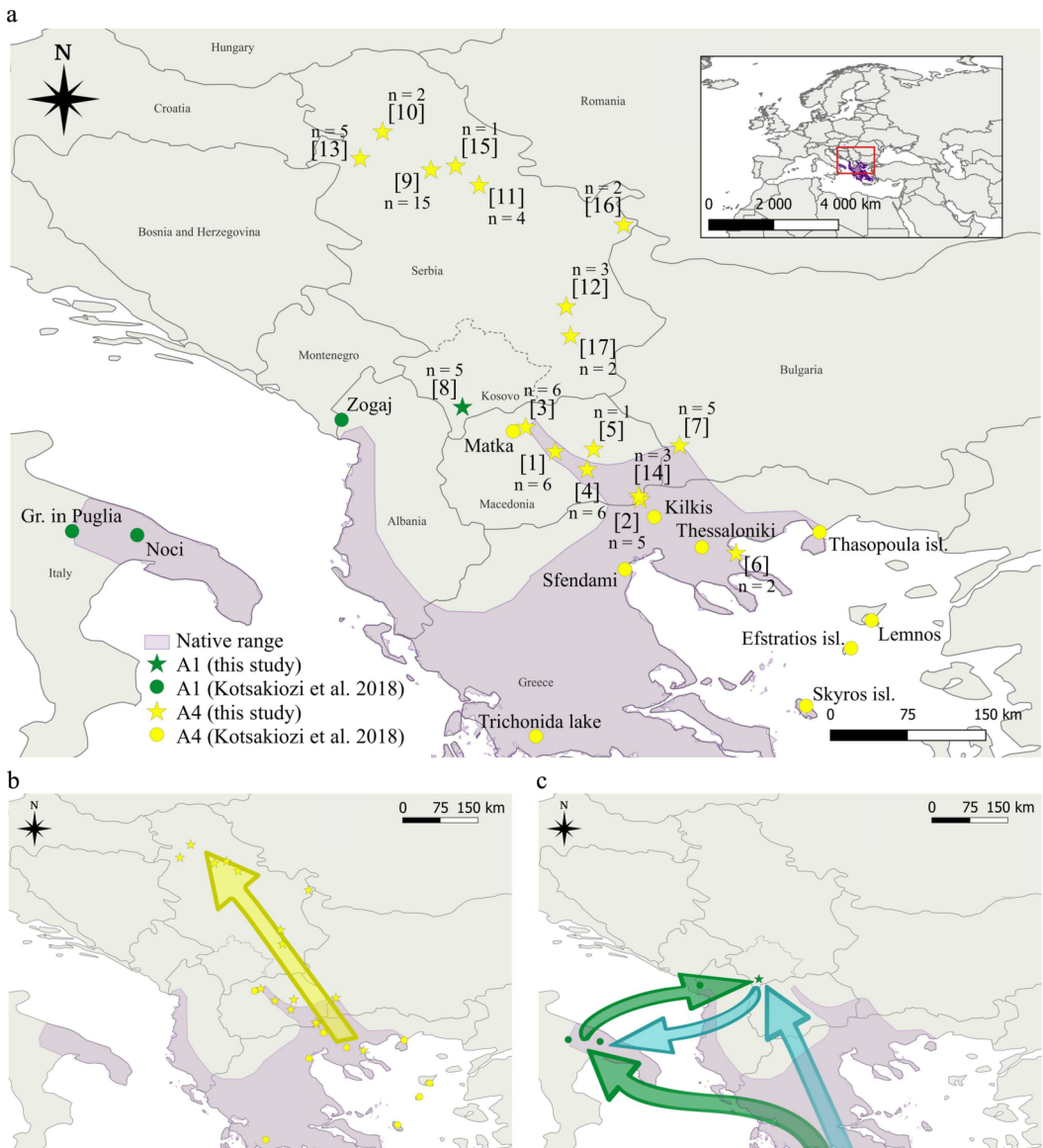


Figure 1. (a) Map of the sampling locations of *Mediodactylus kotschy* from this study (star-shaped), and Kotsakiozi et al. (2018) (dot-shaped) of the subclades A1 and A4. The colour assigned to each locality depends on the subclade it belongs to, according to both the BI and ML phylogenies (fig. 2 and supplementary fig. S1). The newly sampled populations are assigned a locality number (see table 1 for correspondence) and population sample size. The currently known native distribution of *M. kotschy* is shown in purple (Böhme et al., 2009; modified according to Kotsakiozi et al., 2018). (b) Map of the hypothesized colonization pattern of *Mediodactylus kotschy skopensis*. (c) Map of the two possible colonization pathways of *Mediodactylus kotschy kotschy* in Prizren (Kosovo). In green it is shown the hypothesis of an Italian origin, possibly linked to the annexation by Italian troops of that region during World War II. In blue it is shown the hypothesis that the population from Prizren originated from the mainland Balkans, then spreading to southern Italy.

(Ajtić, 2009; Urošević et al., 2021), with even more populations discovered in recent years, such as in Sremska Mitrovica, Leskovac and Negotin (Urošević et al., 2021, 2023). However,

these Serbian populations are more likely to be recently introduced (Ajtić, 2009; Urošević et al., 2016, 2021, 2023). Since no genetic studies have been assessed yet, the early historical

introduction hypothesis (Ajtić, 2009) – even if unlikely since they would have been noticed earlier (Urošević et al., 2021) – still needs to be tested. The Prizren population was also presumed to be native or historical, only based on how well-established it is (Ajtić and Tomović, 2001). Also, Urošević et al. (2016) noticed that this population is isolated from the rest of the species native range, but at the time the Serbian region was lacking data.

No negative impacts of *M. kotschy* over native taxa are known up to date (Urošević et al., 2016). However, its ecological effects have not been evaluated yet, hence this study falls within the scope of surveillance and preliminary assessment of *M. kotschy* patterns of introduction.

In this study, we aim to clarify the phylogenetic relationships among the Serbian populations of *M. kotschy*, as well as between the Serbian populations and those within the native and introduced range in other parts of Europe, to ultimately assess their origin. For that, we have sampled several individuals of *M. kotschy* across nine different Serbian populations. Then, we assessed the phylogenetic relationships based on three mtDNA markers (COI, cytb, 16S rRNA) under a Maximum Likelihood (ML) and Bayesian Inference (BI) approaches.

Material and methods

Samples

Tail tips of 78 individuals assigned as *Mediodactylus kotschy* from 25 populations (13 from Serbia, 10 from North Macedonia, one from Greece and one from Bulgaria) were collected and preserved in 96% ethanol. Forty of these samples were from the Herpetological Collection of the Institute for Biological Research “Siniša Stanković” (Džukić et al., 2017), while 38 samples (35 from Serbia and 3 from North Macedonia) were collected specifically for this study since 2020. These samples had significant conservation quality differences, due to distinct sampling dates and/or preservation methods (fig. 1a, table 1).

DNA extraction, amplification, and sequencing

For each sample, DNA extraction was performed using the high-saline method (Sambrook et al., 1989). DNA presence

was tested on gel electrophoresis (0.8% agarose + gel red, 300V). Amplification by Polymerase Chain Reaction (PCR) was performed for each of the mitochondrial markers (COI, cytb, and 16S). The primers used and the PCR conditions are available in supplementary tables S1 and S2. Amplification success was assessed on a 2% gel electrophoresis. All amplified fragments were sequenced in a Sanger sequencer and deposited in GenBank (accession numbers to be added upon acceptance of the manuscript).

Phylogenetic analysis

Raw sequences were visually checked and cleaned using Geneious Prime v.2023.1.2 (Biomatters Ltd). A total of 174 sequences of *M. kotschy* were retrieved from GenBank and added to our dataset, most of them generated by Kotsakiozi et al. (2018) (see supplementary table S3). Phylogenetic analyses were based on the three mitochondrial fragments, using four species of the genus *Tarentola* (*T. mauritanica*, *T. augustimentalis*, *T. boehmei*, and *T. deserti*) as outgroups. Alignment was performed using MAFFT v.7.490 (Katoh, 2002; Katoh and Standley, 2013), under default parameters.

To determine the best-fit partitioning scheme and DNA substitution model(s), we used a partitioned analysis for multi-gene alignments implemented in IQ-TREE v.2 (Chernomor et al., 2016). The best partition set and models are described in supplementary table S4.

Phylogenetic analyses were performed using Bayesian (MrBayes v. 3.2; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and Maximum Likelihood (RaxML-NG; Kozlov et al., 2019) inferences (BI and ML, respectively). In BI, all analyses started with randomly generated trees and ran for 8×10^6 , with sampling at intervals of 100 generations. Two independent runs were performed and burn-in of 25% was determined upon checking stabilisation of the log likelihood using Tracer v.1.7.2 (Rambaut et al., 2018). The Maximum Likelihood analysis was done with a total of 200 starting trees (100 random and 100 parsimony) and 1000 bootstraps for branch support, estimated after checking for tree convergence with the *bsconverge* function. Computation of branch support was performed using the Transfer Bootstrap Expectation support metric (TBE; Lemoine et al., 2018), which is supposedly more appropriate for very large trees.

Trees were visualized using FigTree v.1.4.4 (Rambaut, 2009).

Haplotype networks

In order to investigate the haplotype diversity and structure within each marker, we built a haplotype network for each mtDNA locus, using a Median Joining approach (Bandelt et al., 1999). To construct these haplotype networks, we used the software PopART (Version 1.7; Leigh and Bryant, 2015), with the parameter epsilon set to 0.

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Table 1. List of the newly sequenced *Mediodactylus kotschy* samples, with the corresponding geographical coordinates as well as the country and locality. Each locality has been assigned a number, the same as in Fig. 1a. For each individual and each mitochondrial marker, a '+' indicates if sequencing was successful and used in the study.

Individual	Country	Locality [n°]	Latitude	Longitude	Clade	16S	Cytb	COI
ND1	North Macedonia	Veles [1]	41.716456	21.772297	A4	+	/	+
ND2	North Macedonia	Veles [1]	41.716456	21.772297	A4	+	/	+
ND3	North Macedonia	Veles [1]	41.716456	21.772297	A4	+	/	/
ND4	North Macedonia	Veles [1]	41.716456	21.772297	A4	+	/	+
ND5	North Macedonia	Veles [1]	41.716456	21.772297	A4	+	/	+
ND7	North Macedonia	Veles [1]	41.713585	21.779427	A4	+	/	/
ND8	North Macedonia	Star Dojran [2]	41.185148	22.720003	A4	+	+	+
ND9	North Macedonia	Star Dojran [2]	41.185148	22.720003	A4	+	/	+
ND10	North Macedonia	Star Dojran [2]	41.185148	22.720003	A4	+	/	+
ND11	North Macedonia	Star Dojran [2]	41.185148	22.720003	A4	+	/	+
ND12	North Macedonia	Star Dojran [2]	41.185148	22.720003	A4	+	/	+
ND13	North Macedonia	Skopje [3]	42.000349	21.398625	A4	/	+	/
ND14	North Macedonia	Skopje [3]	41.987558	21.445093	A4	+	/	/
ND15	North Macedonia	Skopje [3]	41.987558	21.445093	A4	+	/	/
ND16	North Macedonia	Skopje [3]	41.987558	21.445093	A4	+	/	/
ND17	North Macedonia	Skopje [3]	41.987558	21.445093	A4	+	/	+
ND18	North Macedonia	Skopje [3]	41.987558	21.445093	A4	+	/	/
ND19	North Macedonia	Negotino [4]	41.482285	22.092406	A4	+	/	/
ND21	North Macedonia	Štip [5]	41.745394	22.199869	A4	/	+	/
ND22	North Macedonia	Negotino [4]	41.518623	22.128148	A4	+	/	/
ND23	North Macedonia	Negotino [4]	41.518623	22.128148	A4	+	/	/
ND24	North Macedonia	Negotino [4]	41.518623	22.128148	A4	+	/	/
ND25	North Macedonia	Negotino [4]	41.518623	22.128148	A4	+	/	/
ND26	North Macedonia	Negotino [4]	41.518623	22.128148	A4	+	/	/
ND27	Greece	Stravros [6]	40.591138	23.783425	A4	+	/	/
ND28	Greece	Stravros [6]	40.591138	23.783425	A4	+	/	/
ND29	Bulgaria	Kresna Gorge [7]	41.783141	23.155196	A4	+	+	+
ND30	Bulgaria	Kresna Gorge [7]	41.783141	23.155196	A4	+	/	/
ND31	Bulgaria	Kresna Gorge [7]	41.783141	23.155196	A4	+	/	+
ND32	Bulgaria	Kresna Gorge [7]	41.783141	23.155196	A4	+	/	/
ND34	Kosovo	Prizren [8]	42.209657	20.741676	A1	+	+	+
ND35	Kosovo	Prizren [8]	42.209657	20.741676	A1	+	/	+
ND36	Kosovo	Prizren [8]	42.209657	20.741676	A1	+	+	+
ND37	Kosovo	Prizren [8]	42.209657	20.741676	A1	+	+	/
ND38	Kosovo	Prizren [8]	42.209657	20.741676	A1	+	+	+
ND41	Serbia	Novi Sad [10]	45.261026	19.853714	A4	+	/	+
ND42	Serbia	Novi Sad [10]	45.261026	19.853714	A4	+	/	+
ND43	Serbia	Smederevo [11]	44.668147	20.927865	A4	+	/	/
ND45	Serbia	Belgrade [9]	44.804773	20.473959	A4	+	+	+
ND46	Serbia	Belgrade [9]	44.81165	20.456363	A4	+	/	+
ND47	Serbia	Niš [12]	43.323278	21.895081	A4	+	/	/
ND48	Serbia	Niš [12]	43.323278	21.895081	A4	+	/	/
ND49	Serbia	Niš [12]	43.323278	21.895081	A4	+	/	+
ND50	Serbia	Sremska Mitrovica [13]	44.966675	19.604609	A4	+	/	+
ND51	Serbia	Sremska Mitrovica [13]	44.966675	19.604609	A4	+	/	/
ND52	Serbia	Sremska Mitrovica [13]	44.966675	19.604609	A4	+	/	+
ND53	Serbia	Sremska Mitrovica [13]	44.966675	19.604609	A4	+	/	/
ND54	Serbia	Sremska Mitrovica [13]	44.966675	19.604609	A4	+	/	/
ND55	North Macedonia	Nov Dojran [14]	41.224184	22.700829	A4	+	/	+
ND56	North Macedonia	Nov Dojran [14]	41.224184	22.700829	A4	+	+	+
ND57	North Macedonia	Nov Dojran [14]	41.224184	22.700829	A4	+	+	+
ND58	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	/	+
ND59	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	/	+

Table 1. (Continued.)

Individual	Country	Locality [n°]	Latitude	Longitude	Clade	16S	Cytb	COI
ND60	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	/	+
ND61	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	/	+
ND62	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	+	+
ND63	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	+	+
ND64	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	/	+
ND65	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	/	+
ND66	Serbia	Belgrade [9]	44.838572	20.391341	A4	+	+	+
ND67	Serbia	Pančevo [15]	44.882168	20.666578	A4	+	+	+
ND68	Serbia	Belgrade [9]	44.811666	20.457879	A4	+	+	+
ND69	Serbia	Belgrade [9]	44.811666	20.457879	A4	+	/	+
ND70	Serbia	Belgrade [9]	44.811666	20.457879	A4	+	+	+
ND71	Serbia	Belgrade [9]	44.81165	20.456363	A4	+	+	+
ND72	Serbia	Smederevo [11]	44.66807	20.927771	A4	+	/	+
ND73	Serbia	Smederevo [11]	44.66807	20.927771	A4	+	+	+
ND74	Serbia	Smederevo [11]	44.66807	20.927771	A4	+	+	+
ND75	Serbia	Negotin [16]	44.229258	22.533537	A4	+	+	+
ND76	Serbia	Negotin [16]	44.229258	22.533537	A4	+	+	+
ND77	Serbia	Leskovac [17]	42.9988	21.942375	A4	+	+	+
ND78	Serbia	Leskovac [17]	42.9988	21.942375	A4	+	+	+

Results

A total of 1675 bp for 72 sequences generated in our study, with an additional 174 previously published sequences of the *Mediodactylus kotschy* species-complex (Kotsakiozi et al., 2018; Kasapidis et al., 2005), were used to assess the phylogeny of the Serbian *M. kotschy* (table 1, supplementary table S3). Out of these, 370 bp derived from cytb, 689 bp from COI and 616 bp from the 16S gene.

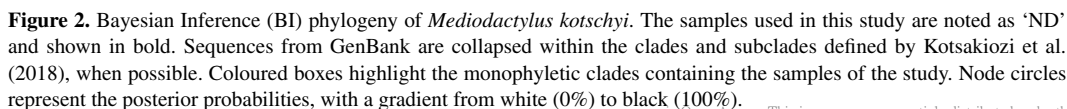
The two phylogenetic approaches showed similar topologies, as well as similar node supports (fig. 2 and supplementary fig. S1). Overall, the obtained Bayesian topology is slightly different from the BI phylogeny from Kotsakiozi et al. (2018). Although we also identified five major clades of *M. kotschy*, our results suggest that clades E and A are sisters, and that Clade D is the most basal one, with high node support (fig. 2). As for the ML tree, it shows a topology more congruent with Kotsakiozi et al. (2018), with only clade B being sister to clade C instead of clade A (supplementary fig. S5). What is important to highlight is that within Clade A (corresponding to *M. kotschy*) the topologies obtained here are similar and highly supported.

We found that subclade A4 contains all our samples, except the population from Prizren which forms a monophyletic group and a sister to the Southern Italian populations.

The haplotype networks show results congruent with the phylogenies with a clear separation of the distinct subclades (fig. 3). The close relationship between the Prizren and Italian population is evident for the three markers (fig. 3a). Within the subclade A4, our samples show four haplotypes for 16S, and only three haplotypes for the two other markers. If we restrict to Serbia, this diversity decreases to three haplotypes for 16S and does not change for the two other markers.

Discussion

This study confirms the genetic affiliation of the Central Balkan populations to *M. kotschy*, specifically to *M. kotschy skopjensis* (Karaman, 1965), according to the revised taxonomy of the species provided by Kotsakiozi et al. in 2018 (fig. 2 and supplementary fig. S1). This genetic identification contributes to the update of the subspecies’ geographical range.



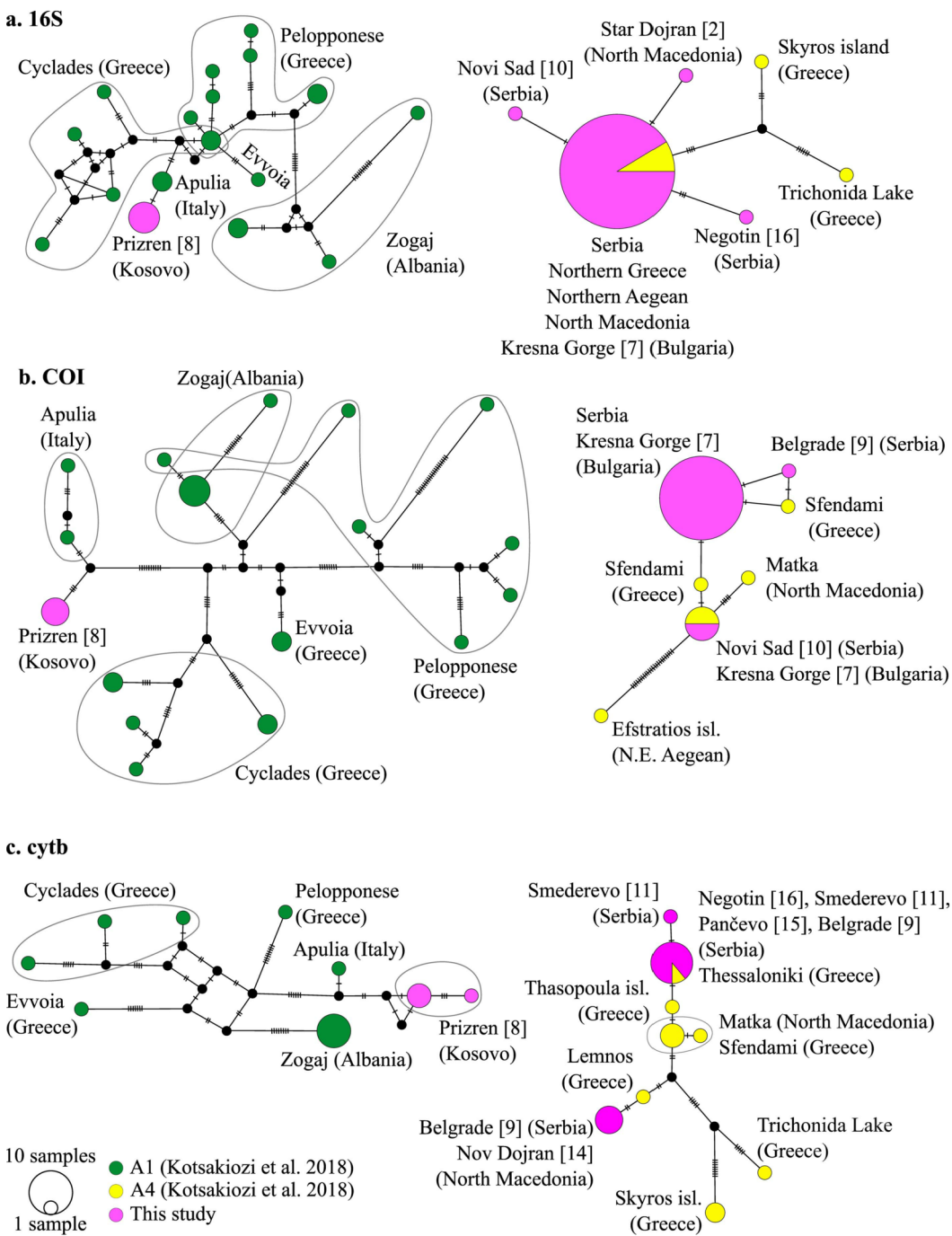


Figure 3. Haplotype networks of all samples from clade A used in this study, for (a) 16S, (b) COI, and (c) cyt b. Haplotypes coloured as “This study” correspond to the new sequences obtained in this study.

The taxonomical assignment of these specimens had already been suggested by Urošević et al. (2021, 2023) based on pholidosis data. More precisely, the Serbian populations from Niš, Sremska Mitrovica, Zemun, Pančevo, Belgrade, Smederevo, Negotin and Leskovac were identified as belonging to the subspecies *M. kotschy bibrani*. However, our molecular data fails to agree with this morphological assessment, suggesting that most of the inland of Central Balkans is populated by *M. kotschy skopjensis* (Karaman, 1965; subclade A4), except for Prizren that seems to be populated exclusively by *M. kotschy kotschy* (Steindachner, 1870; subclade A1), according to the currently accepted taxonomy (Kotsakiozi et al., 2018).

The clustering of the Prizren specimens (subclade A1) with those from Italy (where the species is introduced), suggests a recent origin for this population, most likely human-mediated. The haplotype networks also support the close relationship between these two regions (fig. 3). The observed pattern could be linked to the annexation by Italian troops of that region during World War II (fig. 1c). However, the direction of the introduction remains uncertain. Also, the population from Prizren could have been originated from the mainland Balkans rather than Italy (fig. 1c), but the lack of sampling in the Ionian and South Adriatic coastal regions does not allow this hypothesis to be tested.

Considering that the southern Aegean and northern Greece regions are the current centres of genetic diversity of *M. kotschy* (Kotsakiozi et al., 2018), it seems wise to consider they are also the cradle of this gecko diversification and speciation. Despite the geographical distances separating them, the low genetic differentiation among our specimens of *M. kotschy skopjensis* suggests that these populations originated from northern Greece / North Macedonia region, from where they headed to the north of the Balkan Peninsula very recently, possibly human-mediated (fig. 1b). Interestingly, contrary to *M. kotschy kotschy* that seems to be

solely restricted to Prizren, *M. kotschy skopjensis* is found to be spread all over the centre of the Balkan Peninsula. Belgrade is the capital city of Serbia, with intensive ingoing and outgoing human exchanges since the Antiquity (Urošević et al., 2016), which could lead to the coexistence of distinct subspecies of *M. kotschy* but more sampling would be necessary to assess that. The non-observation in our study of potential contact zones between subclades across Serbia might be related to the sampling gap north of Prizren (see population 8 in fig. 1a). Therefore, extensive sampling across known and other populations is paramount, which could unravel unexpected genetic diversity and the identification of potential contact zones between subspecies.

As like much of the Mediterranean Basin, the Balkan Peninsula situated at the cross-road of the European and Asian parts of the Roman – and Ottoman – Empires, has been a hotspot of human-exchanges for centuries (Urošević et al., 2016, 2021). Hence, this constant interaction among humans and their surrounding biodiversity, explains many of the recently observed herpetofaunal introductions across the Balkans (e.g., Mizsei et al., 2016; Panjković et al., 2021; Gvozdenović and Ikić, 2022). Not surprisingly, this seems to be the cause of the observed phylogeographical pattern of *M. kotschy* in Serbia, as already suggested by previous works (Ajtić and Tomović, 2001; Ajtić, 2009; Urošević et al., 2016; Kotsakiozi et al., 2018). The effect of climate change, which amplifies the urban “heat island effect” could also be an important factor that facilitates population establishment in thermophilic species (Battles and Kolbe, 2019). Another example of a successfully introduced reptile across the Balkans is *Podarcis muralis*. In Slovenia, more precisely in Ljubljana, the species is thought to be introduced from both Serbia and Italy through the railways (Santos et al., 2019). In Reni (Ukraine), multiple sympatric lineages were found, closely related to samples from Serbia, Romania and Bulgaria.

(Oskyrko et al., 2020). These multiple introductions are also thought to be railway-mediated. Likewise, railways were proposed as the most probable way of introduction of *M. kotschy* in Serbia (Urošević et al., 2021).

To conclude, this study clarifies that *Mediodactylus kotschy* is indeed the only species of the genus introduced to the Central Balkans. Moreover, the genetic confirmation of *M. kotschy skopjensis* in Serbia, North Macedonia and Bulgaria adds important information regarding the currently known geographic distribution of this subspecies. However, the observed phylogeographic pattern suggests that Serbian populations are the result of recent introductions (possibly human-mediated), which originate from northern Greece and North Macedonia. Nevertheless, since some of the introduction pathways are unclear, further sampling and the use of additional faster-evolving genetic markers (e.g., RAD-seq) should be considered. Finally, the confirmation that *M. kotschy skopjensis* and *M. kotschy kotschy* are indeed not native in Serbia deserves further investigation/monitoring regarding its possible deleterious effects on native taxa.

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